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Short Communication

# *Achromobacter denitrificans* SP1 produces pharmaceutically active 25C prodigiosin upon utilizing hazardous di(2-ethylhexyl)phthalate

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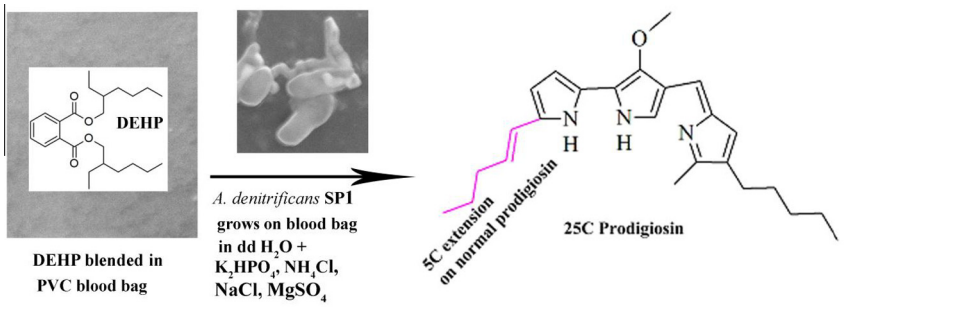
## HIGHLIGHTS

- A novel therapeutically significant prodigiosin from *A. denitrificans* SP1 demonstrated.
- *A. denitrificans* SP1 produced 25C prodigiosin *in situ* and *ex situ*.
- Characterized an unusual 25C prodigiosin analog with a 5C extension on the typical 20C prodigiosin.
- Hazardous DEHP bioremediated into a pharmaceutically significant drug.
- *In silico* studies showed activation of Jak 3, Zap 70 kinases.

## GRAPHICAL ABSTRACT

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## ABSTRACT

This first report describes the purification and identification of an orange-red pigment produced by *Achromobacter denitrificans* strain SP1 (isolated from sewage sludge heavily contaminated with plastics) during its growth in a simple basal salt medium supplemented with the hazardous di(2-ethylhexyl)phthalate (DEHP) blended in PVC blood bag (*in situ*) or free DEHP (*ex situ*) as carbon source. The cell-bound pigment was elucidated, characterized at molecular level, and described as an unusual 25C prodigiosin analog for the first time. At laboratory conditions (in flasks), the dry cell mass was 75.2 mg/g blood bag, which upon extraction yielded 7.1 mg prodigiosin; at this stage the pH of the medium was dropped from 7.2 to 3.5. Considering its pharmaceutical importance, taking 10 known prodigiosins as controls, this 25C prodigiosin was subjected to molecular docking studies, showed comparable and promising binding efficiencies with the crucial molecular human targets like cycloxygenase-2, ZAP-70 kinase and Jak-3 kinase.



## 1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) – a hazardous plasticizer and proven environmental contaminant known to cause various disorders in humans – is being used ruthlessly in almost all polyvinyl chloride (PVC) plastics, especially those in medical use (Heudorf et al., 2007). We for the first time demonstrated that the mycelial fungi can efficiently degrade DEHP blended in a commercial PVC plastics *in situ*, i.e., blood bag (BB) (Pradeep and Benjamin, 2012; Pradeep et al., 2013). Many bacteria are also known to degrade various phthalates (Liang et al., 2008). However, no report is available in literature which describes the production

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of a pharmaceutically-significant secondary metabolite as a by-product, during the process of microbial remediation of any phthalate. Prodigiosin is a versatile secondary metabolite of great interest, owing to its drugabilities such as anti-cancer, immunosuppressive, anti-diabetic, anti-rheumatic, anti-bacterial, anti-fungal and anti-parasitic properties (Kim et al., 2004). *In silico* simulation studies is an efficient tool in predicting the drugability of the molecules by elucidating the molecular interactions with target receptors (Sarath Josh et al., 2014a,b), by partly replacing laborious and expensive mechanistic *in vitro* studies.

In the present study, the efficacy of *Achromobacter denitrificans* SP1 (a novel isolate described from our laboratory) is explored for producing a novel prodigiosin analog in a simple basal salt medium (BSM), supplemented with free DEHP (*ex situ*) or PVC-BB pieces containing intact DEHP (*in situ*) as sole source of carbon and energy. Based upon this, the specific objectives of the present study were: (a) production of prodigiosin from *A. denitrificans* SP1 at laboratory conditions, (b) molecular characterization of prodigiosin, (c) *in silico* molecular docking studies to assess the drugability of 25C prodigiosin with human receptors like cycloxygenase-2, ZAP-70 kinase and Jak-3 kinase.

## 2. Methods

### 2.1. Organism and growth conditions

The bacterium, *A. denitrificans* SP1 (GenBank Accession No. HQ645935; MTCC No. 5710, a patent deposit from our laboratory), isolated from heavily plastics polluted sewage sludge collected from the Canoly Canal; flowing through Kozhikode City, Kerala, India (11.2500 N; 75.7667 E) was cultured in a simple BSM containing (g/L) $K_2HPO_4$, 1.0; NaCl, 1.0; $NH_4Cl$, 0.5 and $MgSO_4$, 0.4 (initial pH, 7.2; and supplemented with DEHP blended) (*in situ* cultivation) BB (Pradeep and Benjamin, 2012; Pradeep et al., 2013). Commercially available HL haemopack BB (Batch No. HO 30419B) manufactured by the Hindustan Latex Ltd., Thiruvananthapuram, India was used as model PVC plastics, this commercial product was plasticized with ~33.5% (w/w) DEHP (Sarath Josh et al., 2012). The DEHP-bound BB (1 g BB/10 mL BSM) was used for *in situ* cultivation, while commercially available liquid DEHP (VWR-BDH-Prolabo, India) was used for *ex situ* cultivation (2.5 mM DEHP in BSM). The culture was incubated at 28 °C in a temperature-controlled shaker at 150 rpm (Scigenics Biotech, India) with initial pH 7.2. The present study has reference to *in situ* cultivation.

*A. denitrificans* SP1 was cultured in 10 mL of BSM supplemented with 1 g BB pieces as sole source of carbon, which was inoculated with 100 μL ($2.92 \times 10^7$ colony forming units) seed culture. BSM with no carbon source was maintained as control. At an interval of 3 days, the culture (whole flask) was taken for various assays. The bacterial cells bound to BB pieces were separated by stirring on a magnetic stirrer for 5 min; employing this cell suspension, the growth (optical density, OD) of *A. denitrificans* SP1 was measured spectrophotometrically at $\lambda_{600}$ (Elico-BL200 Bio-spectrophotometer, India). The cell suspension as harvested above was centrifuged at 9000×*g* at 4 °C for 10 min in a refrigerated centrifuge (Rota 4R-V/FM, Plasto Crafts, India), the pellet so obtained was weighed out to calculate the biomass. The pH of medium (whole flask) was measured using a digital pH meter (Systronics 335, India) at an interval of 3 days up to 15 days. The biofilm formed by *A. denitrificans* SP1 on the surface of BB pieces after cultivating it in BSM–BB medium was analyzed using the SEM (Hitachi, SU 6600, Japan).

### 2.2. Purification and molecular characterization of prodigiosin

The pigmented (orange-red) cell pellet was harvested at an interval of 3 days of growth from BSM–BB medium. To 1 g cell pellet, 3 mL acetone was added, sonicated with an output wattage of 15 for 3 min (Qsonica, LLC, XL-2000 series, USA), until the entire pigment was transferred from the cells into the solvent, and centrifuged at 8000×*g* for 10 min. Supernatant containing pigment was used for further purification. The concentrated pigment in acetone was purified using silica gel (60–120 mesh size) column; 5% ethyl acetate in *n*-hexane was used as the mobile phase for eluting the pigment from the column. The pigment fractions obtained after column chromatography was pooled and air-dried; about 3 mg pigment was dissolved in 500 μL acetone. Of this, 5 μL was spotted on a silica gel GF 254 coated glass plate and developed with methanol:chloroform (2:1) solvent system.

The absorption spectrum of pigment in acetone was scanned spectrophotometrically in the range of 400–800 nm. Characteristic peak of prodigiosin corresponding to $\lambda_{max}$ was measured. The air-dried pigment was pelleted with potassium bromide (KBr) and analyzed using Jasco FTIR Series (Japan). The relative intensity of transmitted light was measured against the wave length of absorption in the region of 400–4000/$cm^{-1}$. For LC–MS analyses; sample was dissolved in acetonitrile, and 10 μL was analyzed in API-4000 Q trap LC–MS/MS. Operating conditions were as follows: 4500 ionization voltage, 350 °C ionization temperature, ionization mode ESI positive, MS C18 (50 × 4.6 mm) column used, 90:10 (acetonitrile:water) mobile phase, and injection volume was 10 μL with a flow rate of 0.5 mL/min. NMR characterization ($^1$H NMR, $^{13}$C NMR) was performed using the pulse program zg30 with a spectral width of 8278.146 Hz using Bruker Advance 400 MHz NMR spectrophotometer (Germany) with 5 mm probe in $CDCl_3$ solvent.

### 2.3. Computational methods

Selected ligand binding domain (LBD) structures of target receptors were imported from the protein data bank (PDB) directly to the molecular docking software (Glide). The target receptors addressed in this study were: 1CX2 [Cycloxygenase-2 (Prostaglandin synthase-2) complexed with a selective inhibitor, SC-558], 1YVJ (crystal structure of the Jak3 kinase domain complexed with a staurosporine analog), and 1U59 (crystal structure of the ZAP-70 kinase domain complexed with staurosporine). Final optimizations and minimizations (refinement) were performed as described in the Schrödinger Protein Preparation Wizard (PrepWizard). This preparation protocol added hydrogen, built side chains and loops with missing atoms, optimized the H-bonding network and performed a restrained minimization to get the final refined structure of proteins for docking studies. The grid boxes were generated by selecting the co-crystallized ligand on the LBD, and these glide grids were used for the molecular docking with new ligands (by replacing co-crystallized ligand), as we described recently (Sarath Josh et al., 2014a,b). Ten known structures of prodigiosin analogs were retrieved from PubChem database (for convenience, designated by id number) as controls for comparing the binding efficiencies with that of the 25C prodigiosin analog produced by *A. denitrificans* SP1, which were: (**1**) (2Z,5Z)-3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2-yl)methylidene]-5-pyrrol-2-ylidenepyrrole; (**2**) (2E,5Z)-3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2-yl)methylidene]-5-pyrrol-2-ylidenepyrrole; (**3**) (2E,5E)-3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2-yl)methylidene]-5-pyrrol-2-ylidenepyrrole; (**4**) (2Z,5E)-3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2yl) methylidene]-5-pyrrol-2-ylidenepyrrole; (**5**) (2E,5E)-3-methoxy-5-pyrrol-2-ylidene-2-[(5-undecyl-1H-pyrrol-2-yl)methylidene] pyrrole; (**6**) 12-[(Z)-[(5Z)-3-methoxy-5-pyrrol-2-ylidenepyrrol-2-ylidene]methyl]-10-(2-methylpropyl)-13-azabicyclo[9.2.2] tetradeca-1(14),11-diene; (**7**) (2Z,5Z)-3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2-yl)methylidene]-5-pyrrol-2-ylidenepyrrole hydrobromide; (**8**) (2Z,5Z)-3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2-yl)methylidene]-5-pyrrol-2-ylidenepyrrole hydrochlo-

ride; (**9**) 3-methoxy-5-pyrrol-2-ylidene-2-[(5-undecyl-1H-pyrrol-2-yl)methylidene] pyrrole; and (**10**) 3-methoxy-2-[(5-methyl-4-pentyl-1H-pyrrol-2-yl)methylidene]-5-pyrrol-2-ylidenepyrrole; in addition to 25-C prodigiosin produced by *A. denitrificans* SP1. The structures of selected ligands were drawn in Maestro, Schrödinger Suite 2012 and generated the 3D structures with minimized energy. The prepared glide grid of each ligand was individually docked to the LBD of the target receptor using Glide dock-XP mode, the flexible dock. Subsequently, the best binding pose of the ligand to the LBD with maximum glide score (*G* score, with *minus* sign) and glide energy (*G* energy) was generated for each ligand.

## 3. Results and discussion

### 3.1. Growth profile

This is the first report on the isolation and characterization of a prodigiosin-analog produced by a phthalate utilizing organism. Researchers tried to formulate economically cheap media for the production of prodigiosin (Giri et al., 2004). In the present study, a simple and cheap medium [(BSM supplemented with DEHP either in free form (*ex situ*) or PVC-BB (*in situ*)] was used for the cultivation. Upon *in situ* cultivation, *A. denitrificans* SP1 grew well by forming microfilm so as to stick to the BB surface. It was observed that prodigiosin producing *Serratia marcescens* can easily attach to hydrophobic surfaces in its natural habitat than non-prodigiosin producing species (O'Rear et al., 1992). Pigment formation was started on day 3 during *in situ* growth. It was observed that the absorbance increased steadily and attained the maximum on day 12 ($\lambda_{600}$ = 1.84) (Fig. S1A). The biomass was increased gradually up to day 12 during the growth of *A. denitrificans* SP1 on pieces of BB (1 g) in flask containing 10 mL BSM–BB; subsequently it slightly decreased by day 15. The biomass showed a maximum of 75.2 mg/g BB on day 12, corresponding pigment produced was 7.1 mg, i.e., 7.1 mg prodigiosin obtained per gram BB (Fig. S1B). The pH dependent growth profile of *A. denitrificans* SP1 in BSM–BB medium was monitored up to 15 days (Fig. S1C). The pH decreased from the initial value of 7.2–4.0 in a week; in fact, the decrease in pH was started on day 3. Prodigiosin producing *A. denitrificans* SP1 showed strong adherence on the surface of BB in BSM (Fig. S2). Usually, diffusion of phthalates from plastics occurs at sluggish rate, while speedy surface diffusion happens due to surface colonization by microorganisms and further metabolic breakdown of phthalates by specific enzymes (Pradeep and Benjamin, 2012). This adherence can be considered as the primary phase of biofilm formation (Reynolds and Fink, 2001). Gradually, *A. denitrificans* SP1 utilized the DEHP present in BB, and attained the maximum growth on day 12. Pigment production was found to be growth-associated, since it showed a maximum on day 12; subsequently, the growth and pigment production were declined. This may be due to the low acidic environment in the medium; the initial pH (7.2) was decreased to 4.85 in 3 days of continuous growth, which further declined to 3.7 in a couple of weeks. During this period, *A. denitrificans* SP1 consumed almost complete DEHP blended in BB. Previous studies with *S. marcescens* demonstrated that the maximum pigment production was in the stationary or senescence phase of bacterial growth (Williams et al., 1971).

The pigment produced by *A. denitrificans* SP1 may be an adaptive response to the nutrient stress induced by DEHP in BSM; because *A. denitrificans* SP1 was unable to produce any pigment in nutrient broth or in the presence of any other carbon source (including other phthalates) in the growth medium. The pigment production was found to be the maximum at 28 °C, and not observed any pigment in the same culture broth at 37 °C (Giri et al., 2004). Higher rate of agitation caused a small decrease in the production of undecylprodigiosin by *S. marcescens* strain, because of shear force; wherein mass transfer of medium components and oxygen into the cells had a significant role in pigment production (Wei and Chen, 2005). Our study showed small scale production (in flasks) of prodigiosin at laboratory condition, in which about 10% prodigiosin per dry cell mass of *A. denitrificans* SP1 was obtained. This could be enhanced by improving the medium composition and using a suitable bioreactor with optimum growth conditions.

### 3.2. Purification and molecular characterization of prodigiosin

Prodigiosins are localized either as membrane-bound vesicles or as intracellular granules in the cytoplasm (Kobayashi and Ichikawa, 1990). The water-insoluble intracellular pigment produced by *A. denitrificans* SP1 was extracted with acetone by disrupting the cell wall after sonication. The water insoluble prodigiosin from *S. marcescens*, the known producer of prodigiosin, was also extracted with acetone (Cang et al., 2000). For column purification, the solvent system comprised of *n*-hexane and ethyl acetate. Initial elution was done with *n*-hexane. The polarity of the solvent was increased by adding ethyl acetate. Pigment was separated in 95% *n*-hexane:5% ethyl acetate solvent fraction. TLC profile of the purified pigment in acetone showed a single spot with an $R_f$ value 0.67 in the methanol:chloroform (2:1) solvent system. Pigment in acetone was scanned with spectrophotometer in the range of 400–800 nm, and the maximum peak was obtained at $\lambda_{480}$. Prodigiosin produced by *S. marcescens*, was red in acid solution with an absorption maximum at 535–540 nm, whereas it was yellow-orange in alkaline solution with an absorption maximum at 470 nm (Allen, 1967). We observed bright-red coloration to the pigment with the maximum intensity upon growth on day 12 with absorption maximum at 480 nm; at this stage, the pH of the medium declined to acidic.

The FT-IR characterization showed wave numbers ($cm^{-1}$) with characteristic vibrations at 3289 (N–H str aromatic), 2823 and 2852 (C–H str aliphatic) 1650 (N–H str aromatic) and 1539 $cm^{-1}$ (C–O str ether in $-OCH_3$). Upon LC–MS characterization, the molecular mass of the purified compound was 391. The NMR spectral characterization of the pigment (both $^1H$ NMR and $^{13}C$ NMR) is given in Table S1 (Supporting information). The position of each proton is indicated in $^1H$ NMR, and that of each carbon is represented in $^{13}C$ NMR spectra. Using IR, MS and NMR spectral characterization, the pigment produced by *A. denitrificans* SP1was identified as 25C prodigiosin. This 25C prodigiosin had a molecular formula of $C_{25}H_{33}N_3O$.

### 3.3. Molecular docking

The drugability (ADME/Tox) of 25C prodigiosin was tested using QikProp analysis. The Lipinski Rule of 5 Violations toward suitability of a drug, 25C prodigiosin satisfied four qualities. Of 1712 known drugs, 25C prodigiosin shows significant similarity to Alfacalcidol (88.6%), Tiocarlide (85.59%), Drotaverine (84.52%), Toremifene (82.2%) and Clomiphene (82.16%).

The 25C prodigiosin produced by *A. denitrificans* SP1 is supposed to have the therapeutic properties shown by other prodigiosin analogs. In comparison to known prodigiosin analogs, preliminary *in silico* analyses showed that this novel prodigiosin has promising binding efficiencies with the crucial molecular targets such as cyclooxygenase-2, ZAP-70 kinase and Jak-3 kinase, which further indicate the efficiency of this analog for immunomodulation (these targets were identified after thorough screening of over 100 potential targets). We analyzed the binding efficiencies of 25-C prodigiosin reported herein, and compared it with 10 known prodigiosins (20C, 25C or 27C) as ligands onto the molecular human targets

such as PDB ids: 1CX2, 1YVJ and 1U59 using Glide dock. Even though various parameters like *G* score, *G* energy, H-bonding, ligand efficiency, etc. were analyzed; for simplicity of data presentation, *G* score – being the prime inference of molecular docking – has been focused in this *in silico* study for describing the docking efficiency of ligands. Details of 10 known prodigiosins, their PubChem id, *G* score upon binding with PDB ids: 1CX2, 1YVJ and 1U59 are summarized in Table S2. Comparison of *G* scores; i.e., the maximum obtained with already reported prodigiosins and that obtained with the present 25C prodigiosin from *A. denitrificans* SP1 with 1CX2, 1YVJ and 1U59 are represented in Fig. S3.

*3.3.1. Molecular interactions of 1CX2 with prodigiosin analogs*

Regarding the 25C prodigiosin reported in the present study, oxygen atom in the $-OCH_3$ group formed 1 H bond with the amino acid (*aa*) residue Tyr 355 (*G* score −5.21 kcal/mol) resided in the LBD of the receptor 1CX2. Similar binding pattern was observed for the known prodigiosin with PubChem id: 5386692 (*G* score of −8.578); but with PubChem id: 6711250, the nitrogen atom in the pyrrole ring formed 1 H bond with Tyr 355 in the LBD, i.e., the highest *G* score (−8.64) of a prodigiosin with 1CX2. The PubChem compounds 5377753 and 5351169 showed only van der Waals interactions with the *aa* residues in the LBD (with *G* scores −5.49 and −4.60, respectively). Regarding PubChem: 5464066, the –NH group in the pyrrole ring formed one π–π interaction with His 90 and 1 H bond with the Leu 352 in the LBD, which resulted in the lowest *G* score (−1.58). However, with PubChem: 65239, the –NH group in the pyrrole ring formed 1 H bond with Leu 352 in the LBD (*G* score −5.97); while with PubChem: 5359273, Arg 120 formed a π–cation interaction in the LBD with *G* score −3.65. Cyclooxygenases (COX 1 and 2) are the major enzymes involved in the synthesis of prostaglandins, the major mediators of inflammation, pain and increased body temperature (hyperpyrexia) (McGettigan and Henry, 2006). The COX-2 is associated with inflamed tissues and is an important target for the treatment of rheumatoid arthritis. In contrast to the isoform COX-1, use of drugs that inhibit COX-2 is of great importance due to low risk of peptic ulceration (McGettigan and Henry, 2006). Therefore we selected COX-2 as one of the targets. Employing the software GOLD, Krishna et al. (2013) analyzed the role of prodigiosins in anti-inflammatory function and found that prodigiosin interacted with Leu 321 and Tyr 324 in the LBD of COX-2. The computational ligand binding interaction suggested >45% higher fitness score value for prodigiosin to that of cycloprodigiosin (Krishna et al., 2013). In the present study using Glide, the 25-C prodigiosin showed interaction with Tyr 355 in the LBD of COX-2, and showed a moderate docking score (−5.21 kcal/mol) and −63.53 kcal/mol glide energy.

*3.3.2. Molecular interactions of 1U59 binding with prodigiosin analogs*

The 25C prodigiosin from *A. denitrificans* SP1, the oxygen atom in the $-OCH_3$ group formed 1 H bond with Ala 417 in the LBD and obtained a *G* score of −5.39. Similar molecular interactions were detected for PubChem: 5351169 (*G* score −6.36), PubChem: 6711250 (*G* score −5.96) and PubChem: 5386692 (*G* score −6.29). Regarding PubChem: 50985825, the –NH group in the pyrrole ring formed 1 H bond with Cys 346 in the LBD with a *G* score of −4.09; while PubChem: 5464066 showed a *G* score of −5.82, where –NH group in one of the tripyrrole rings formed 1 H bond with Gly 418 and another ring formed 1 H bond with Ala 417 in the LBD, whereas upon interaction with PubChem: 5377753, the –NH groups in two pyrrole rings formed 2 H bonds with Ala 417, and the –NH group in the third ring formed another 1 H bond with Gly 418 in the LBD (*G* score −7.38). The –NH group in one of the tripyrrole rings of PubChem: 5359273 formed 1 H bond with Leu 344 in the LBD (*G* score −5.09). Jak3 mediates signal transduction *via* the gamma common chain of lymphokine surface receptors. Selective Jak3 inhibitors have shown utility in several autoimmune disorders, allograft rejection during transplantation, acute lymphoblastic leukemia, type 1 diabetes, rheumatoid arthritis, allergic and asthmatic diseases (Podder and Kahan, 2004). It was reported that a prodigiosin analog PNU156804 selectively inhibited γ-chain cytokine activation of Jak3 and subsequent serine kinase pathways *in vitro*, and prolonged heart allograft survival in rats (Stepkowski et al., 2002). In the present study, the binding of 25C prodigiosin from *A. denitrificans* SP1 with the LBD of Jak3 showed promising results; it established 1 H-bond with the *aa* Asp 967 (*G* score of −6.59 and *G* energy −45.04). Only two other analogs, i.e., PubChem: 5464066 and 5359273 showed higher docking scores (−7.71 and −6.78, respectively) than this 25C prodigiosin.

*3.3.3. Molecular interactions of 1YVJ binding with prodigiosin analogs*

The 25C prodigiosin from *A. denitrificans* SP1 showed the *G* score −6.59; which was comparable to that of PubChem: 5386692 (*G* score −6.35) and PubChem: 5377753 (*G* score −5.39), i.e., the oxygen atom in the $-OCH_3$ group formed 1 H bond with the Asp 967 in the LBD of 1YVJ. PubChem: 6711250 (*G* score −4.68) and PubChem: 5351169 (*G* score −6.02) showed similar bindings, i.e., the oxygen atom in the $-OCH_3$ group formed 1 H bond with Leu 905 in the LBD; the PubChem compounds 6725 (*G* score −5.39) and PubChem: 50985825 (*G* score −5.37) established only van der Waals interactions with the *aa* residues in the LBD. The binding of PubChem: 65239 (*G* score −1.91) and PubChem: 5359273 (*G* score −6.78) showed that the –NH group in the pyrrole ring established 1 H bond with Pro 906 and Leu 905 in the LBD, respectively; while with PubChem: 5464066 (*G* score −7.71), the –NH groups in two of the pyrrole rings formed 2 H bonds – one with Pro 906 and another with Leu 905 in the LBD. Zap-70 is a cytoplasmic protein tyrosine kinase that plays a significant role in the events involved in the initiating of T-cell responses by the antigen receptor (Wang et al., 2010). Inhibition of Zap-70 activity specifically aims at T-cell responses, leaving innate immunity intact. Such an inhibitory molecule can be used to prevent allograft rejection or T-cell-mediated autoimmunity (Hirabayashi et al., 2009). Though few ZAP-70 inhibitors have been described, their specific activities on T cells or *in vivo* have not been demonstrated. Therefore, a highly specific, cell permeable and small molecule that inhibits Zap-70 is of great importance (Wang et al., 2010). From the present study; compared to other prodigiosin analogs, 25-C prodigiosin from *A. denitrificans* SP1 established 1 H-bond with Ala 417 in LBD, and showed comparable binding efficiency (*G* score of −5.39 and *G* energy −45.13) with Zap-70 kinase.

Under these experimental conditions, *G* scores between *minus* 7 and *minus* 13 are considered as moderate and excellent, respectively. Thus, the binding efficiency of the 25C prodigiosin is moderate with regard to the selected receptors: 1CX2, 1U59 and 1YVJ. This efficiency is comparable to that of different known pharmaceutically active prodigiosins, which further highlights the pharmaceutical importance of the novel 25C prodigiosin produced by *A. denitrificans* SP1. Thus, prodigiosin and its analogs are emerging up as new class of therapeutics with proven claims on anti-cancer, immunosuppressive, anti-diabetic, anti-rheumatic, anti-bacterial, anti-fungal and anti-parasitic properties. Moreover, demands for natural products are increasing day-by-day; hence identification of novel microbes with potentials for the production of pharmaceutically active natural pigments and optimization of production conditions need to be extended, especially with a perspective to remediate the hazardous pollutants into such valuable products.

## 4. Conclusions

The 25C prodigiosin analog reported from *A. denitrificans* SP1 (MTCC No. 5710) is the first of this kind, produced by any organism

in the presence of a phthalate. The strategy demonstrated herein is efficient for the bioremediation of DEHP (*in situ* or *ex situ*), and concomitant production of prodigiosin. Like known prodigiosin analogs, this 25C prodigiosin showed favorable docking score and energy upon binding with protein targets such as 1CX2, 1U59 and 1YVJ. The therapeutic significance of 25C prodigiosin should not be restricted to the tested molecular targets; also other targets could be explored through *in silico*, *in vitro* and *in vivo* studies.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.08.077.

## References

Allen, E.G., 1967. Conditions of the colour change of prodigiosin. Nature 216, 929–931.

Cang, S., Sanada, M., Johdo, O., Ohta, S., Nagamatsu, Y., Yoshimoto, A., 2000. High production of prodigiosin by *Serratia marcescens* grown on ethanol. Biotechnol. Lett. 22, 1761–1765.

Giri, A.V., Anandkumar, N., Muthukumaran, G., Pennathur, G., 2004. A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. BMC Microbiol. 4 (1), 11.

Heudorf, U., Mersch-Sundermann, V., Angerer, J., 2007. Phthalates: toxicology and exposure. Int. J. Hyg. Environ. Health 210 (5), 623–634.

Hirabayashi, A., Mukaiyama, H., Kobayashi, H., Shiohara, H., Nakayama, S., Ozawa, M., Miyazawa, K., Misawa, K., Ohnota, H., Isaji, M., 2009. Structure–activity relationship studies of 5-benzylaminoimidazo[1,2-c]pyrimidine-8-carboxamide derivatives as potent, highly selective ZAP-70 kinase inhibitors. Bioorg. Med. Chem. 17, 284–294.

Kim, H.M., Han, S.B., Lee, C.W., Lee, K.H., Park, S.H., Kim, H.C., Kim, Y., 2004. Prodigiosin Composition for the Treatment of Rheumatic Arthritis. U.S. Patent No. 20,040,127,547.

Kobayashi, N., Ichikawa, Y., 1990. Separation of the prodigiosin-localizing crude vesicles which retain the activity of protease and nuclease in *Serratia marcescens*. Microbiol. Immunol. 35 (8), 607–614.

Krishna, P.S., Vani, K., Prasad, M.R., Samatha, B., Surya, N.S.V.S.S., Bindu, L.H., Charya, M.A.S., Shetty, P.R., 2013. In-silico molecular docking analysis of prodigiosin and cycloprodigiosin as COX-2 inhibitors. SpringerPlus 2 (1), 1–6.

Liang, D.-W., Zhang, T., Fang, H.H., He, J., 2008. Phthalates biodegradation in the environment. Appl. Microbiol. Biotechnol. 80 (2), 183–198.

McGettigan, P., Henry, D., 2006. Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of the observational studies of selective and nonselective inhibitors of cyclooxygenase 2. JAMA- J. Am. Med. Assoc. 296, 1633–1644.

O'Rear, J., Alberti, L., Harshey, R.M., 1992. Mutations that impair swarming motility in *S. marcescens* 274 include but not limited to those affecting chemotaxis or flagellar function. J. Bacteriol. 174, 6125–6137.

Podder, H., Kahan, B., 2004. Janus kinase 3: a novel target for selective transplant immunosuppression. Expert Opin. Ther. Target. 8, 613–629.

Pradeep, S., Benjamin, S., 2012. Mycelial fungi completely remediate di (2-ethylhexyl) phthalate, the hazardous plasticizer in PVC blood storage bag. J. Hazard. Mater. 235–236, 69–77.

Pradeep, S., Faseela, P., Sarath Josh, M.K., Balachandran, S., Sudha Devi, R., Benjamin, S., 2013. Fungal biodegradation of phthalate plasticizer *in situ*. Biodegradation 24 (2), 257–267.

Reynolds, T.B., Fink, G.R., 2001. Bakers' yeast, a model for fungal biofilm formation. Science 291 (5505), 878–881.

Sarath Josh, M.K., Pradeep, S., Balachandran, S., Sudha Devi, R., Vijayalakshmi Amma, K.S., Benjamin, S., 2012. Temperature- and solvent-dependent migrations of di(2-ethylhexyl)phthalate, the hazardous plasticizer from commercial PVC blood storage bag. J. Polym. Res.. http://dx.doi.org/10.1007/s10965-012-9915-4.

Sarath Josh, M.K., Pradeep, S., Adarsh, V.K., Vijayalekshmi Amma, K.S., Sudha Devi, R., Balachandran, S., Sreejith, M.N., Abdul Jaleel, U.C., Benjamin, S., 2014a. *In silico* evidences for the binding of phthalates onto human estrogen receptor α, β subtypes and human estrogen-related receptor γ. Mol. Simulat. 40, 408–417.

Sarath Josh, M.K., Pradeep, S., Vijayalekshmi Amma, K.S., Balachandran, S., Abdul Jaleel, U.C., Doble, M., Spener, F., Benjamin, S., 2014b. Phthalates efficiently bind to human peroxisome proliferator activated receptor and retinoid X receptor α, β, γ subtypes: an in silico approach. J. Appl. Toxicol. 34 (7), 754–765.

Stepkowski, S.M., Erwin-Cohen, R.A., Behbod, F., Wang, M.-E., Qu, X., Tejpal, N., Nagy, Z.S., Kahan, B.D., Kirken, R.A., 2002. Selective inhibitor of Janus tyrosine kinase 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin. Blood 99, 680–689.

Wang, H., Kadlecek, T.A., Au-Yeung, B.B., Goodfellow, H.E.S., Hsu, L.Y., Freedman, T.S., Weiss, A., 2010. ZAP-70: an essential kinase in T-cell signaling. Cold Spring Harb. Perspect. Biol. 2, a002279. http://dx.doi.org/10.1101/cshperspect.a002279.

Wei, Y.-H., Chen, W.-C., 2005. Enhanced production of prodigiosin-like pigment from *Serratia marcescens* SMΔR by medium improvement and oil-supplementation strategies. J. Biosci. Bioeng. 99 (6), 616–622.

Williams, R.P., Gott, C.L., Qadri, S.H., Scott, R.H., 1971. Influence of temperature of incubation and type of growth medium on pigmentation in *Serratia marcescens*. J. Bacteriol. 106 (2), 438–443.